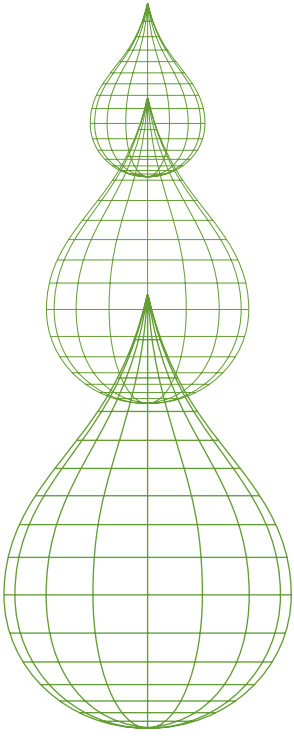
Proseek® Multiplex 96×96

USER MANUAL



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1. Introduction

Proseek® Multiplex$^{96\times96}$ from Olink Bioscience is a diverse product line of reagents for scalable immunoassays enabling simultaneous measurement of 92 protein biomarkers in 1 µL sample volume. The Proseek platform is designed for ease of use and offers enhanced analytical performance, analysis of complex matrices, as well as improvement in assay throughput over conventional immunoassays.

To get you started, Proseek Multiplex$^{96\times96}$ reagents come as a convenient all-in-one kit format with an optimized protocol.

2. Principle of the assay

2.1 TECHNOLOGY AND ASSAY FORMAT

The Proseek reagents are based on PEA, a Proximity Extension Assay technology[1], in which 96 oligonucleotide-labeled antibody pairs are allowed to bind to their respective protein targets in the sample. A PCR reporter sequence is formed by a proximity-dependent DNA polymerization event and is subsequently detected and quantified using real-time PCR. The assay is performed in a homogeneous 96-well format with no need for washing steps.

Proseek Multiplex assay procedure employs three core steps:

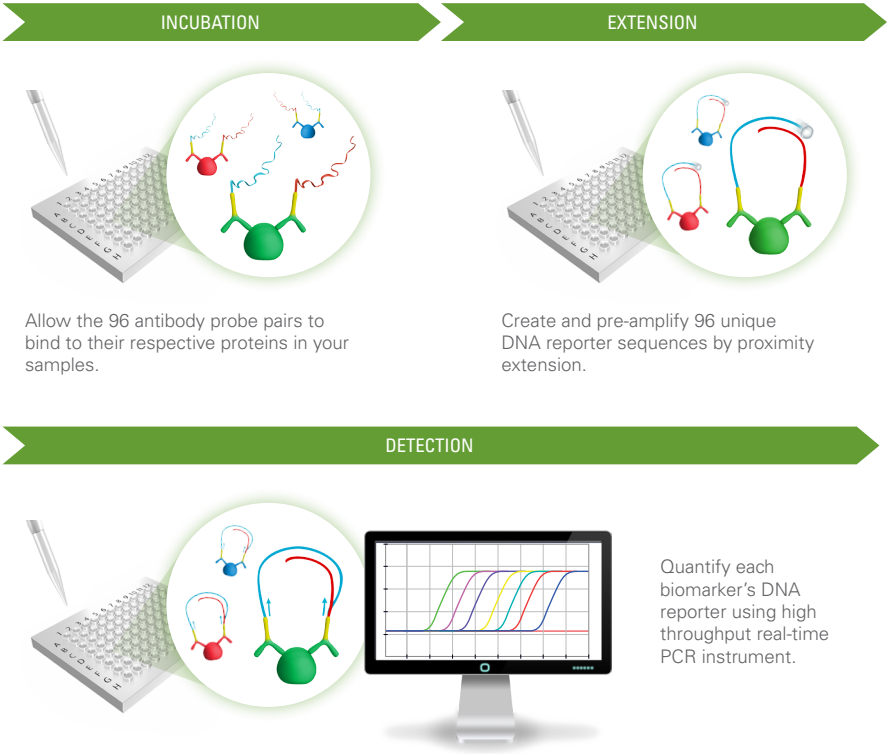


Fig 1. Assay procedure.

3. Reagents and equipment

3.1 REAGENTS SUPPLIED

Each Proseek Multiplex 96×96 kit contains reagents for 96 wells, sufficient for 90 samples and 6 controls. The reagents are supplied in three individual boxes. Storage temperature and expiry date for the components are stated on the outer label of each box.

3.1.1 PROSEEK MULTIPLEX PROBE KIT 96×96 (STORE AT +4°C)

- Incubation Solution
  Contains components needed for the incubation reaction
- A-probes
  Contains 96 antibody probes labeled with A oligos
- B-probes
  Contains 96 antibody probes labeled with B oligos

3.1.2 PROSEEK MULTIPLEX DETECTION KIT 96×96 (STORE AT -20°C)

- PEA Solution
  Contains components needed for the extension reaction
- PEA Enzyme
  For extension of A and B probes which are bound to their target
- PCR Polymerase
  For pre-amplification of the extension product created by the PEA enzyme
- Detection Solution
  Contains components needed for the detection reaction
- Detection Enzyme
  For qPCR amplification
- Primer Plate
  96-well plate with ready-to-use primers for amplification of extension product

3.1.3 PROSEEK MULTIPLEX CONTROLS (STORE AT -20°C)

- Interplate Control
  For normalization between runs
- Negative Control
  For determination of background levels
- Incubation Stabilizer
  For stabilization of the incubation reaction

3.2 REQUIRED CONSUMABLES (NOT SUPPLIED)

- Pipette tips (filter is required)
- Microcentrifuge tubes (1-1.5 mL)
- Centrifuge tube (> 11 mL)
- 8-well strips with lids
- 96-well PCR plate (à 0.2 mL)
- Multi-channel pipette reservoir
- Adhesive plastic film (heat-resistant)
- High purity water (sterile filtered, MilliQ® or similar)
- 96.96 Dynamic Array™ Integrated Fluidic Circuit (IFC), (Fluidigm Corporation, catalogue number BMK-M96.96)

3.3 REQUIRED EQUIPMENT (NOT SUPPLIED)

- Pipettes (covering the range from 1 µL to 1000 µL)
- Multi-channel pipettes (recommended range 1-10 µL, 50-100 µL or 50-200 µL)
- Vortex
- Centrifuge for plates
- Microcentrifuge for tubes
- Freezing block (-20°C) for enzyme handling
- Thermal cycler with:
  - Heated lid
  - Temperature range from 50°C to 95°C
  - Validated for 0.1mL volumes - important
  - 96-well format (recommended)

  (Olink has tested ABI 2720 and ABI Veriti® 96-Well Thermal Cycler)
- Refrigerator or cold room (+2°C to +8°C)
- Fluidigm BioMark™ or BioMark™ HD System
- Fluidigm IFC controller HX

3.4 SOFTWARE FOR ANALYSIS

Each Proseek Multiplex $^{96\times96}$ experiment will generate 9216 data points. It is advisable to use a multivariate statistical software for data analysis. We recommend the GenEx software developed by MultiD Analyses AB. GenEx offers an easy-to-use Olink Wizard to guide you through the different steps of data preprocessing followed by a wide variety of statistical analysis such as hierarchical clustering methods, principal components analysis, ANOVA tests and more. For more information please contact support@olink.com.

3.5 DOWNLOADS

The list of proteins can directly be imported into the Fluidigm Analysis software as a .plt file. Download the Detector Setup.plt file corresponding to your panel at www.olink.com/products/proseek-multiplex/downloads/data-analysis-files.

For data normalization without the Olink Wizard for GenEx, please download the DataPreprocessing.xlsx file corresponding to your panel at www.olink.com.

3.6 TECHNICAL CONTROLS

- Each Proseek Multiplex 96x96 kit contains four internal controls; Incubation Control 1, Incubation Control 2, Extension Control and Detection Control. The internal controls are included in the assay reagents, and hence added to each sample to be tested.
  - The two Incubation Controls are two control immunoassays measuring spiked-in non-human antigens. These controls measure the variation in all three steps of the assay; Incubation, Extension and Detection.
  - The Extension Control is an antibody labeled with both pair of oligonucleotides needed for amplification. The Extension Control is not dependent on the proximity of two different antibodies, thus not affected during Incubation. This control is used for normalization and compensates for the variation between samples in the Extension and Detection steps of the assay.
  - The Detection Control is a synthetic oligonucleotide that monitors the Detection step. This control is not affected during Incubation or Extension.
- The three replicates of Interplate Control are used for normalization and compensates for possible variation between runs.
- The three replicates of Negative Control are used to calculate the lower limit of detection (LOD) for each protein.

4. Assay considerations

4.1 SAFETY

Follow general laboratory safety procedure such as using gloves, safety goggles and protective clothing when performing the experiments. Handle and dispose of hazardous sample material according to local regulations.

4.2 PCR TECHNOLOGY

PCR technology is sensitive to contaminations; perform the Detection step in a post-PCR room, separate from the room where the Incubation and Extension steps (to step 19, page 13) are performed. Maintain and calibrate your PCR and BioMark instruments regularly.

4.3 PIPETTING TECHNIQUES

It is advisable to use a multi-channel pipette in the reagent transfer steps. Use filter pipette tips to avoid contamination. Change pipette tips between all sample and reagent transfer steps to avoid cross-contamination. Maintain and calibrate your pipettes regularly.

4.4 SAMPLE PREPARATION

To reduce sample-handling time during the experiment, samples can be aliquoted in 8-well strips or 96-well plate prior to the start of the experiment.

4.5 SAMPLE MATERIAL

Proseek Multiplex$^{96\times96}$ has been validated on EDTA plasma (all panels) and serum (Oncology and Inflammation) samples. A range of additional sample types are compatible with the technology; such as citrate plasma, heparin plasma, tissue and cell lysates and saliva. Different sample matrices can affect the detection of specific proteins. For more information on sample material, please see the data package corresponding to each panel. Guidelines on tissue lysate buffers are available in Appendix 2. For questions, please contact support@olink.com.

5. Assay protocol

5.1 EXPERIMENTAL DESIGN

It is important to plan your experimental design properly to get the data you need to answer your questions. Decide how many samples, replicates and controls you will need to get the data you want. Consult a statistician to be on the safe side prior to running your study.

5.2 PLATE LAYOUT

Prior to running the Proseek Multiplex$^{96\times96}$ assay, plan the distribution of samples across the plate. It is important to place the Negative Control and the Interplate Control in the last 6 wells according to Figure 2. Proseek Multiplex $^{96\times96}$ kit is designed for 90 samples, three replicates of Negative Control and three replicates of Interplate Control. For analysis of less than 90 samples, please pipette replicates of selected samples.

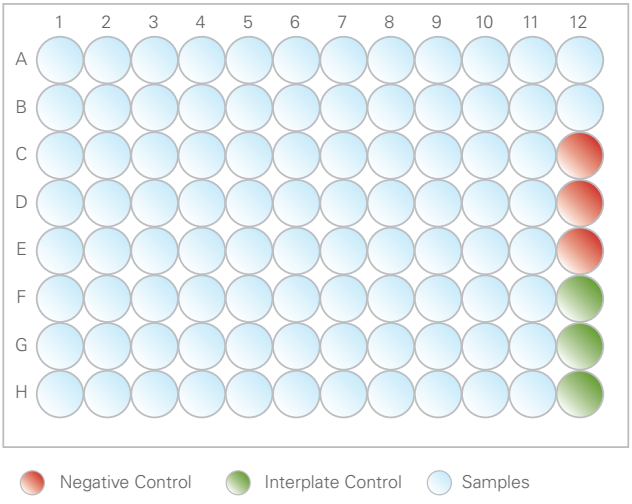


Fig 2. Plate layout.

5.3 PEA PROGRAM

Create a *PEA program* on the thermal cycler with the following conditions. Enable the heated lid function.

Extension	50°C	20 min	
Hot start	95°C	5 min	
PCR Cycle	95°C	30 s	×17
	54°C	1 min	
	60°C	1 min	
Maintain the reaction at	10°C	∞, hold	

5.4 OLINK PROTEIN EXPRESSION 96×96 PROGRAM

Program the Fluidigm BioMark System with the following steps. Name the program *Olink Protein Expression 96×96 program.*

Thermal mix	50°C	120 s	
	70°C	1800 s	
	25°C	600 s	
Hot Start	95°C	300 s	
PCR Cycle	95°C	15 s	×40
	60°C	60 s	

Verify correct settings:
Application – **Gene Expression**
Passive Reference – **5-Carboxy-x-Rhodamine (abbreviation ROX in Fluidigm software)**
Assay – **single probe**
Probes – **FAM-MGB**

5.5 FLUIDIGM INSTRUCTIONS

For information on the Fluidigm IFC Controller HX and Fluidigm BioMark System, please read through the following User Guides (www.fluidigm.com)

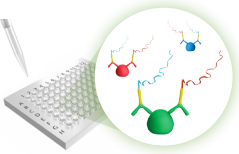
- Fluidigm® IFC Controller User Guide - PN 68000112
- Fluidigm® Real-Time PCR Analysis User Guide - PN 68000088
- Fluidigm® Data Collection Software User Guide - PN 68000127

5.6 PROSEEK MULTIPLEX$^{96\times96}$ PROTOCOL

Before starting:

- Please read the entire Proseek Multiplex$^{96\times96}$ protocol.
- Decide how many samples you will include in the experiment and the number of replicates.
- Use the 96-well plate template in Figure 2 and select a location for each sample.

Day 1:



INCUBATION

1. Thaw samples, vortex and spin down the content at 150 × g, 1 min at room temperature.
2. Thaw the Incubation Stabilizer from the Proseek Multiplex Detection Kit $^{96\times96}$ box, vortex and spin briefly.
3. Thaw the Interplate Control and Negative Control from the Proseek Multiplex Controls box, vortex and spin briefly.
4. Prepare the following *Incubation mix* in a microcentrifuge tube. Vortex and spin each reagent before transfer to the mix.

Incubation mix	per 96-well plate (µL)
Incubation Solution	280.0
Incubation Stabilizer	40.0
A-probes	40.0
B-probes	40.0
Total	**400.0**

Note: *Pipette the Incubation Solution carefully to avoid foaming. Please note that the volumes have been changed from previous version (v.1.3).*

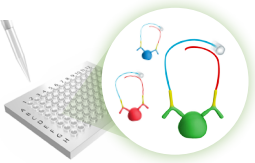
5. Vortex the *Incubation mix* briefly and spin down the content. Transfer 47 µL per well of the *Incubation mix* by using reverse pipetting to an 8-well strip.
6. Use a multi-channel pipette to transfer 3 µL of the *Incubation mix* from the 8-well strip to the bottom of each well of a 96-well plate by using reverse pipetting. Do not change pipette tips. Name this plate *Incubation Plate*.

Note: *Pipette from the uppermost part of the* Incubation mix *to prevent liquid from sticking to the outside of the pipette tip.*

7. Add 1 µL of each sample to the bottom of the well of the *Incubation Plate* according to your plate layout.
8. Add 1 µL of Negative Control to the bottom of each well in position C12, D12 and E12 according to the plate layout in Figure 2.
9. Add 1 µL of Interplate Control to the bottom of each well in position F12, G12 and H12.
10. Seal the *Incubation Plate* with an adhesive plastic film. It is important that all wells are properly sealed, especially around the edges to avoid evaporation of samples. Spin down the content at 150 × g, 1 min at room temperature.
11. Incubate the *Incubation Plate* overnight at +2°C to +8°C.

Day 2:

EXTENSION



12. Turn on your thermal cycler and activate the heated lid function.

13. Thaw the PEA Solution, vortex and spin briefly. Prepare the following *Extension mix* in a centrifuge tube. Use a freezing block when removing the PEA Enzyme and the PCR Polymerase from -20°C and spin down the content briefly before pipetting the enzymes into the mix.

Extension mix	per 96-well plate (µL)
High Purity Water	9385
PEA Solution	1100
PEA Enzyme	55
PCR Polymerase	22
Total	**10 562**

14. Vortex the *Extension mix*.

15. Bring the *Incubation Plate* to room temperature.
Spin down the content at 150 × g, 1 min at room temperature.

16. Pour the *Extension mix* into a multi-channel pipette reservoir.

17. Carefully remove the plastic adhesive film from the *Incubation Plate*.

18. Start a timer set for 5 min and transfer 96 µL of *Extension mix* to each well of the *Incubation Plate* by using reverse pipetting. Do not change pipette tips, place the tips against the upper parts of the well wall. Make sure the tips never come in contact with the contents of the wells.

Note: *Perform steps 18–20 within 5 minutes.*

19. Add a new plastic adhesive film to the *Incubation Plate*. It is important that all wells are properly sealed, especially around the edges to avoid evaporation of samples.

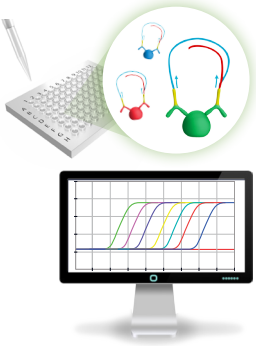
20. Vortex gently and spin down the content at 150 × g, 1 min at room temperature.

21. After the 5 min, place the *Incubation Plate* in the thermal cycler and run the *PEA program* (see section 5.2 for details). The *PEA program* takes approximately 1 h 40 min.

Note: *If your thermal cycler requires a silicon cover for plates covered with plastic film, please use one to avoid evaporation.*

22. Continue with the Detection step or store the Incubation Plate for up to one week at +4°C.

DETECTION



23. Prepare and prime a 96.96 Dynamic Array IFC according to the manufacturer's instructions.

24. Thaw the Primer Plate, vortex and spin at 150 × g, 1 min at room temperature.

25. Thaw the Detection Solution, vortex and spin briefly. Prepare the following *Detection mix* in a microcentrifuge tube. Use a freezing block for the Detection Enzyme and PCR Polymerase and spin down the content briefly before pipetting the enzymes into the mix.

Detection mix	per 96-well plate (µL)
Detection Solution	550.0
High Purity Water	230.0
Detection Enzyme	7.8
PCR Polymerase	3.1
Total	**790.9**

26. Vortex the *Detection mix* and spin briefly. Transfer 95 µL of the *Detection mix* per well to an 8-well strip.

27. Use a multi-channel pipette to transfer 7.2 µL of *Detection mix* to each well of a new 96-well plate by reverse pipetting. Name this plate *Sample Plate*.

28. Remove the *Incubation Plate* from the thermal cycler, vortex and spin down the contents.

29. Carefully remove the plastic film and transfer 2.8 µL from each well of the *Incubation Plate* to the *Sample Plate*.

30. Seal the *Sample Plate* with a new plastic adhesive film, vortex and spin at 150 × g, 1 min at room temperature.

31. Transfer 5 µL from each well of the *Sample Plate* to the primed 96.96 Dynamic Array IFC by using reverse pipetting. Change pipette tips after each sample. Samples are loaded into their respective inlets on the right side of the chip according to Figure 3. See Appendix 1 for a detailed instruction on sample loading.

Note: *For steps 31 and 32, make sure not to leave any inlets empty on the chip.*

32. Gently remove the Primer Plate aluminum sealing to avoid contamination between wells. Transfer 5 µL from each well of the Primer Plate into the inlets on the left side of the chip according to Figure 3 by reverse pipetting. Change pipette tips after each transfer.

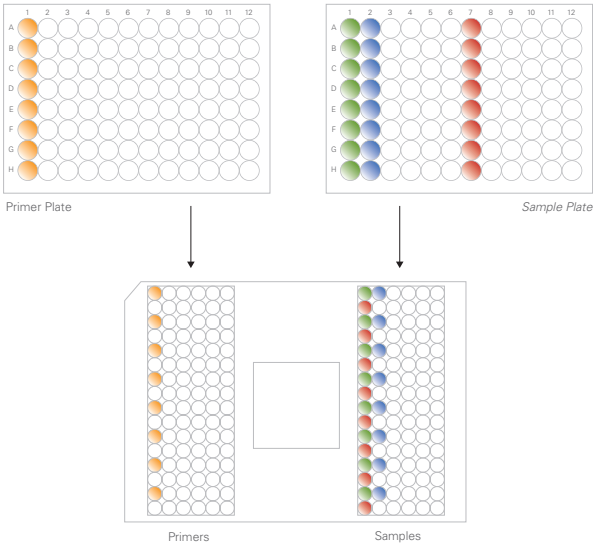


Fig 3. Loading of samples and primers to the 96.96 Dynamic Array IFC.

Note: *The chip should be oriented so that the cut corner of the chip is placed on your upper left side.*

33. Remove any visible bubbles, *e.g.* with a pipette tip or syringe needle and change between each well.

34. Load the chip in the Fluidigm IFC Controller HX according to manufacturer's instructions.

35. Run the *Olink Protein Expression 96×96 Program* in the Fluidigm Biomark Reader according to manufacturer's instructions (See 5.3 for detailed instructions on the *Olink Protein Expression 96×96 Program*).

6. Results and data analysis

6.1 EXPORT THE DATA

Verify the BioMark run using the Fluidigm Real-Time PCR analysis software according to manufacturer's instructions. Export your data to a spreadsheet (see Olink Wizard for GenEx User Guide for a detailed instruction).

6.2 DATA UNITS

The Proseek assay generates Cq values. To even out variation between runs and within run, the data should be normalized using the Extension Control, Interplate Control and a correction factor. The data used for further statistical analysis is in Normalized Protein Expression (NPX) units on log2 scale where a high value corresponds to high protein concentration. For calculating Coefficient of Variation (%CV) between replicate samples, you need to use linear values. Convert your NPX values to linear values by using this formula: 2^(NPX). For normalization of your data, please use the Olink Wizard for GenEx or download the DataPreprocessing.xlsx file corresponding to your panel at www.olink.com.

6.3 OLINK WIZARD FOR GENEX SOFTWARE

Each Proseek Multiplex$^{96\times96}$ experiment will generate 9216 data points. To facilitate data analysis we recommend the Olink Wizard plugin for GenEx. GenEx is a multivariate statistical analysis software developed by MultiD Analyses AB (www.multid.se). The Olink Wizard for GenEx, will guide you through data qualification steps and provide you with Normalized Protein Expression (NPX) values for further statistical analysis.

The GenEx software offers a variety of statistical analysis tools such as hierarchical clustering methods, principal components analysis, ANOVA tests and more. Different visualization tools, including scatter plot, box and whisker plot or bar graph, allow you to rapidly identify major differences across samples and provide you with images for data presentations.

For further information about data analysis, please contact Olink at support@olink.com.

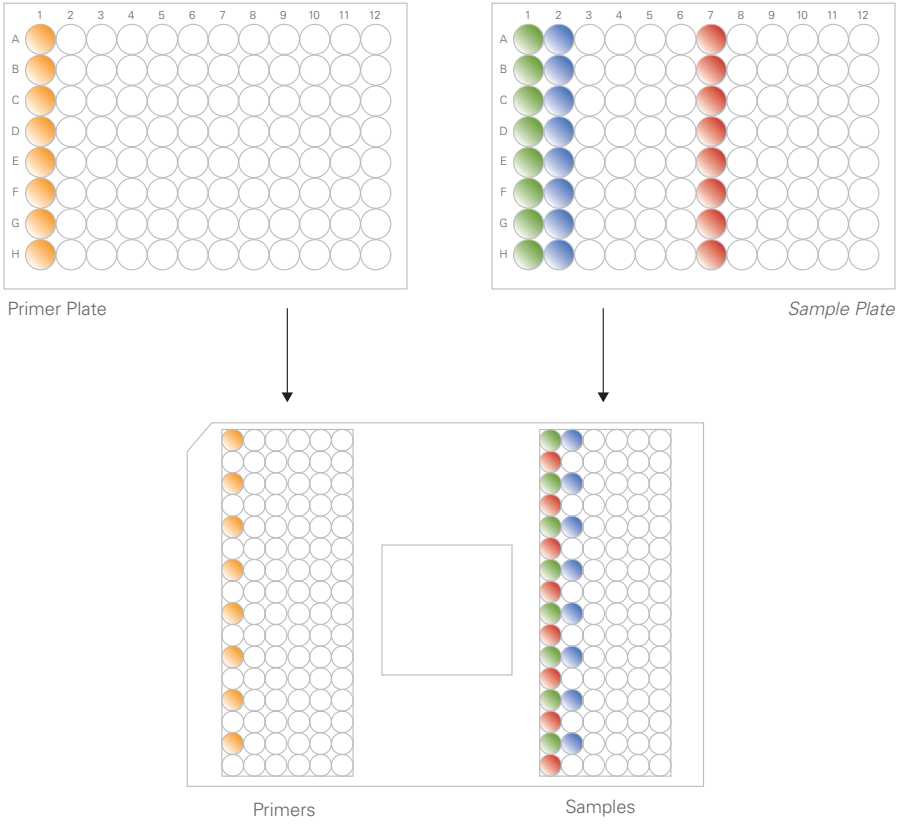
7. References

1. Lundberg, M., *et.al.* Homogeneous antibody-based proximity extension assays provide sensitive and specific detection of low abundant proteins in human blood. *Nucleic Acid Res* 6 June (2011). doi: 10.1093/nar/gkr424.

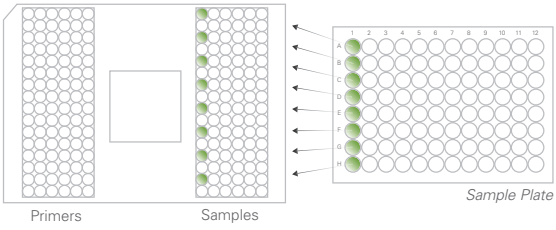
2. Assarsson E., *et.al.* Homogenous 96-Plex PEA Immunoassay Exhibiting High Sensitivity, Specificity, and Excellent Scalability. *PLOS One* 6 April (2014) 9:4. e95192.

Appendix 1

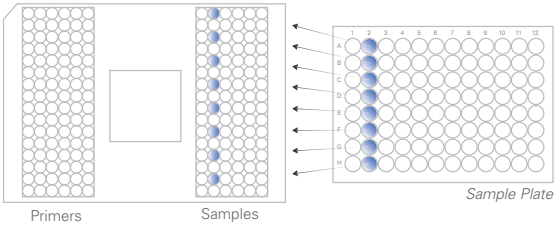
Load samples to the right and primers to the left on the 96.96 Dynamic Array IFC.



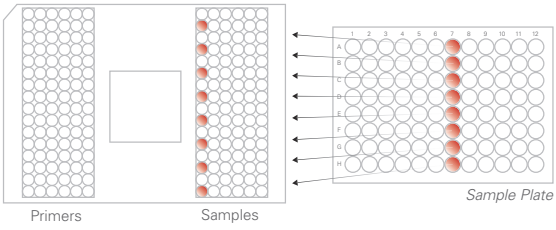
1. Use reverse pipetting. Transfer 5 µL from each well in position 1 A-H (marked in green) to inlets in the first column on the right side of the chip (green). When using an eight-channel pipette every other inlet will be filled according to the image.



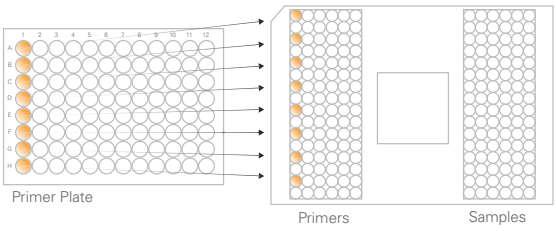
2. Transfer 5 µL from each well in position 2 A-H (blue) to the second column of inlets (blue) according to image. Continue with columns 3-6.



3. Transfer 5 µL from each well in position 7 A-H (red) to inlets in the first column on the right side of the chip (red), start on the second row according to image. Continue with columns 8-12.



4. Transfer 5 µL from each well in the Primer Plate to the inlets on the left side of the chip in the same manner as described in steps 1-3 for *Sample Plate*.



Appendix 2

TISSUE LYSATES

Proseek Multiplex 96×96 is compatible with human tissue lysates. Two different lysis buffers and different tissues such as lung tissue, muscle tissue, endometrial tissue and skin/melanoma tissue have been selected.

Optimization of lysis buffer may be necessary, depending on tissue type. Make sure you have equal amount of tissue in each sample. Determine the total protein concentration in each sample and standardize by diluting high level samples. Samples standardized to approximately 0.5-1 mg/mL based on protein determination carried out using BCA Protein Assay (Lowry) show good result with Proseek Multiplex 96×96. Specific tissues could have very high expression of certain proteins and further dilution may be necessary for the Proseek Multiplex assay, e.g. 1:50 or 1:100.

LYSIS BUFFER 1

- 10x RIPA buffer: (50mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0.1 % Sodium deoxycholate)
- Add fresh to 1x RIPA buffer:
  - Serine protease inhibitor Phenylmethylsulfonyl fluoride (PMSF) to 1 mM
  - Protease Inhibitor cocktail:(final conc 10.4 mM AEBSF, 8 µM Aprotinin, 0.2 mM Leupeptin, 0.4 mM Bestatin, 0.15 mM Pepstatin, 0.14 mM E-64)

For protein determination using BCA (Lowry) method, dilute samples 1:5 in PBS.

LYSIS BUFFER 2

- Bio-Plex Cell lysis kit (cat number 171-304011, Bio-Rad)
- Add fresh to buffer:
  - + Factors 1 and 2 (from the kit)
  - + Protease inhibitor cocktail from Roche (cat number 11 836153001)

For questions, please contact support@olink.com.

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